A comparison of (A9) and (A11) shows that

$$\overline{\rho^2} = \frac{\int_0^\infty Mf(M)\rho^2\,dM}{\bar{M}_w} \qquad (A12)$$

Thus the dependence of the experimentally determined mean square radius of gyration on the molecular weight is rather complicated since it involves the relation of ρ to M. Only in the case of a random coil do we have a simple result as earlier found by Zimm.[36] In this case ρ^2 is proportional to M and it is clear that the size is that of the molecular species having the z-average molecular weight, thus

$$\bar{M}_\rho = \frac{\int M^2 f(M)\,dM}{\bar{M}_w} = M_z \qquad (A13)$$

For spheres, $\rho^2 \sim M^{2/3}$ and we have

$$\bar{M}_\rho = \left[\frac{\int_0^\infty M^{5/3} f(M)\,dM}{\bar{M}_w}\right]^{3/2} < M_z \qquad (A14)$$

For rods, $\rho^2 \sim M^2$ and

$$\bar{M}_\rho = \left[\frac{\int_0^\infty M^3 f(M)\,dM}{\bar{M}_w}\right]^{1/2} > M_z \qquad (A15)$$

The molecular species to which these values refer will be smaller than the z-average for spheres and larger than the z-average for rods. It is apparent from equation A12, however, that the dimension found in light scattering is obtained by the *same* averaging process regardless of the particle shape and is by definition equal to the square root of the z-average of the square of the dimension of the individual scattering centers.

Viscosity.—Writing the Flory–Fox relation in the form

$$\eta_{sp} = \Phi(\overline{r^2})^{3/2}\frac{c}{M} \qquad (A16)$$

it follows that, for a polydisperse system in which the fraction of molecules with molecular weights between $M + dM$ is given by $h(M)\,dM$

$$[\eta] = \frac{\eta_{sp}}{\int_0^\infty Mh(M)\,dM} = \frac{\Phi\int_0^\infty (\overline{r^2})^{3/2} h(M)\,dM}{\int_0^\infty Mh(M)\,dM} \qquad (A17)$$

That is, if we introduce the number average molecular weight in the Flory–Fox relation, we obtain the number average of $(\overline{r^2})^{3/2}$. If, as is more usual, the molecular weight is determined by light scattering, then we obtain by the use of this relation the number average of $(\overline{r^2})^{1/2}$, multiplied by the ratio of M_w/M_n.

Thus the dimensions calculated from angular light scattering measurements and listed in Table IV represent $\sqrt{\overline{L_z^2}}$. Those from the viscosity were obtained on the assumption that the value of Φ given by Flory is only negligibly in error due to the polydispersity of the original polymer solutions employed in the estimation of this parameter.[38] The average dimension tabulated, $\sqrt[3]{(\overline{r^2})_n^{3/2}}$, has been obtained by assuming $M_w/M_n = 1.2$.

(38) S. Newman, W. R. Krigbaum, C. Langier and P. J. Flory, Abstracts, A.C.S. Meeting 124, Chicago, September, 1953.

CAMBRIDGE, MASS.

[CONTRIBUTION FROM THE WESTERN UTILIZATION RESEARCH BRANCH, AGRICULTURAL RESEARCH SERVICE, UNITED STATES DEPARTMENT OF AGRICULTURE]

Reaction-Inactivation of Polyphenoloxidase: Temperature Dependence[1]

BY LLOYD L. INGRAHAM

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Measurements have been made at several temperatures of (a) the initial rates of the polyphenol-catalyzed aerobic oxidation of catechol and (b) the amounts of substrate oxidized when the reaction stops as a result of concurrent inactivation of the enzyme. Two energies of activation were calculated from these data: (a) B for the rate of the enzyme inactivation reaction and (b) A for the rate of the oxidation. The value for A is about the same order of magnitude as those obtained for other oxidative enzymes. The low value found for B suggests that reaction-inactivation is a fairly simple reaction and involves no extensive damage to the protein such as denaturation or dissociation. A mechanism for the reaction inactivation that is consistent with the known experimental facts is proposed. It is postulated that an enzyme–semiquinone complex is an intermediate which may decompose either to (a) reaction products and free enzyme or to (b) an inactive enzyme.

W. H. Miller, *et al.*,[2] have shown that the polyphenoloxidase-catalyzed oxidation of catechol can be followed by measuring the oxidation of ascorbic acid added to the reaction mixture. The semiquinone and orthoquinone formed in the reaction oxidize the ascorbic acid to dehydroascorbic acid and the semiquinone and orthoquinone are reduced back to catechol. In our studies of the prevention of browning of fruit we have been interested in the fact that in the system described above the rate of disappearance of ascorbic acid does not remain constant but gradually decreases until, after 5–10 minutes, the rate becomes zero and no more ascorbic acid is oxidized. This effect has been shown to be due to a disappearance of enzyme activity and has been named "reaction-inactivation,"[3] since it occurs only during the catalyzed oxidation of catechols.

Recent research[4] has shown that the rate of reaction-inactivation of polyphenoloxidase during the

(1) Presented in part at the 123rd A.C.S. meeting, Los Angeles, Calif., March 15–19, 1953.

(2) W. H. Miller, M. F. Mallette, L. J. Roth and C. R. Dawson, THIS JOURNAL, **66**, 514 (1944).

(3) B. J. Ludwig and J. M. Nelson, *ibid.*, **61**, 2601 (1939).

(4) L. L. Ingraham, J. Corse and B. Makower, *ibid.*, **74**, 2623 (1952).

aerobic oxidation of catechol is first-order with respect to the enzyme concentration. This is consistent with the formation of *o*-quinone (or semiquinone), following the relationship

$$Q = \frac{k_0 E_0}{k_I}[1 - e^{-k_I t}] \qquad (I)$$

where Q is the amount of oxidized material formed in time, t. E_0 is the initial enzyme concentration. The rate constants k_0 and k_I are defined by the equation

$$\frac{dQ}{dt} = k_0 E \qquad (II)$$

which expresses the rate of formation of quinone or the rate of disappearance of ascorbic acid and the equation

$$\frac{-dE}{dt} = k_I E \qquad (III)$$

which is the rate of inactivation of the enzyme. Equation I may be derived by integrating equation III and substituting in this the value of E thus obtained in equation II. Integration of equation II will then give equation I.

The present paper describes a study of the temperature coefficient of the rate constant k_I. The value of k_I cannot be measured directly but only in combination with k_0, as can be seen from equation I. In order to obtain a value for energy of activation, B, for k_I alone, two series of measurements were made at various temperatures—first, the limiting amount of quinone oxidized and, second, the initial rate of oxidation. The initial enzyme concentration was held constant during any one series of experiments. We can see from equation I that the limiting amount of oxidized material produced (when $t = \infty$) is equal to $k_0 E_0/k_I$. From the temperature coefficient of this term, we will obtain the difference, $A - B$, where A is the activation energy for k_0. From the temperature coefficient of the initial rate of reaction

$$\left(\frac{dQ}{dt}\right)_{t=0} = k_0 E_0$$

we can calculate the value of A alone. These measurements were made on preparations of enzymes from Newtown Pippin apples partially purified by cold acetone precipitations and also on a sample of commercial mushroom preparation and on a sample of crude mushroom juice (*Psalliota campestris*).

The limiting amount of *o*-quinone produced (or ascorbic acid oxidized) was for all practical purposes attained in 30 minutes for the commercial mushroom and apple enzyme preparations.[4] The cruder preparation of mushroom juice, as is usually the case with crude enzyme preparations, showed less reaction inactivation and as a result the limiting amount of *o*-quinone produced could not be measured even after 60 minutes. Not wishing to use longer experiments, we calculated the limiting amount of *o*-quinone from measurements at 30 and 60 min. and using equation IV[5]

$$\lim_{t\to\infty} Q = \frac{Q_{30}}{2 - \frac{Q_{60}}{Q_{30}}} \qquad (IV)$$

This equation will hold for any two values of Q provided one is taken at twice the time of the other.

Results

The results are given in tabular form in Tables I and II. It is interesting to note in Table I that $\lim_{t\to\infty} Q$ decreased with temperature so that eventually more total oxidation is obtained at low temperatures than at high temperatures. The values of $A - B$ shown in Table II are therefore negative and within experimental errors the same, -3.7 kcal., for the three preparations of enzyme studied, apple enzyme, commercial mushroom enzyme and crude mushroom juice. It is interesting to note that although the amount of reaction inactivation varies with the purity of the enzyme, the heat of activation of the reaction was the same for the two mushroom enzymes of different purity. This suggests that the mechanism of the reaction inactivation is independent of purity of the enzyme. The results obtained for $A - B$ from chronometric data are compared with a value calculated from some meager manometric data of Ludwig and Nelson.[3] They measured the total amount of oxygen the enzyme consumed at two temperatures. It is encouraging that these data from different methods agree within 300 calories.

The value of A is much lower for the apple enzyme, 3.9 kcal., for the mushroom enzymes, 12 kcal. Previous measurements with mushroom juice at slightly different *p*H values and ionic strength have given a much lower value for A of 2.3 kcal.[6] These measurements were made manometrically before the development of the chronometric method. The lower value may be due to the fact that initial rates were not measured. It takes little reaction inactivation to reduce the value of A. Sizer[7] has remarked on the low value of A for polyphenoloxidase (tyrosinase). These new values are more comparable to the values found for other enzymes.

The B value for apple enzyme is about 7.5 kcal./mole and the value for mushroom enzyme around 15.5 kcal./mole. These comparatively low values of B we believe show that the reaction-inactivation reaction must be a fairly simple reaction and not involve any extensive damage to the protein such as denaturation or dissociation.

Discussion

An interesting possibility for the mechanism of the reaction-inactivation consistent with the known facts is shown below

$$E_0 + C \rightleftharpoons E_0C$$
$$E_0C \rightleftharpoons E_RS$$
$$E_RS \longrightarrow E_R + S$$
$$E_RS \longrightarrow E \text{ inactive}$$
$$E_R + O_2 \longrightarrow E_0$$
$$S + \text{ascorbic acid} \longrightarrow C + \text{dehydroascorbic acid}$$

E_0 is the oxidized enzyme, C is the catechol or other substrate, E_R is reduced enzyme and S is semiquinone. The reaction-inactivation will be first order with respect to the total enzyme concentration in

(5) This equation was derived by Dr. H. M. Hughes of the Mathematics Department at the University of California.

(6) B. S. Gould, *Enzymologia*, **7**, 292 (1939).

(7) I. W. Sizer, *Advances in Enzymol.*, **3**, 49 (1943).

TABLE I

VARIATION OF THE ULTIMATE LIMIT OF OXIDATION AND OF THE INITIAL RATE OF OXIDATION WITH TEMPERATURE

Commercial mushroom enzyme[a]			Mushroom juice[a]			Apple enzyme[a]		
Temp.,[b] °C.	$\lim_{t\to\infty} Q$[c]	$(dQ)_{t=0}$[d]	Temp.,[b] °C.	$\lim_{t\to\infty} Qf$	$(dQ)_{t=0}$[d,e]	Temp., °C.	$\lim_{t\to\infty} Q$[c]	$(dQ)_{t=0}$[d]
0.6	1.41	0.36	0.0 (0.3)	13.2	7.4	0.3	81	68
5.5	1.33	.68	4.5 (5.0)	13.1	11	5.0	71	78
10.0	1.17	.92	10.0 (10.5)	11.3	16	10.0	64	88
14.0	1.13	1.36	14.5 (15.3)	9.3	25	14.0	57	92
20.5 (19.8)	0.99	1.80	20.2 (20.6)	8.5	32	20.5	51	106
25.0	.91, 0.88	2.3, 2.6	24.9 (25.0)	7.8	49	25.0	45	124
30.0	.80	4.0	30.3 (29.7)	7.2	60	30.0	43	141
35.0 (35.7)	.70	5.0	(34.7)		81	35.0	37	151

[a] Amounts of substrate (catechol) per 100 ml. of solution: Commercial mushroom enzyme, 20 mg.; mushroom juice, 300 mg.; apple enzyme, 400 mg. [b] The temperatures at which the measurements of initial rates were made are placed in parentheses if different from the temperatures at which $\lim_{t\to\infty} Q$ was measured. [c] Mg. ascorbic acid oxidized per mg. of enzyme at 30 min. [d] Initial rate in catecholase units (1 catecholase of enzyme will oxidize 1.49×10^{-8} moles of catechol per second) per mg. for the commercial mushroom enzyme and per ml. for the other two enzymes. [e] A different sample of enzyme was used for the determination of initial rates from that used for the determination of $\lim_{t\to\infty} Q$. [f] Determined by equation IV. A typical set of data obtained at 4.5° is as follows: Starting initially with 19.9 mg. of ascorbic acid this dropped to 9.8 mg. in 30 min. and to 7.5 mg. in 60 min. Therefore $Q_1 = 19.9 - 9.8 = 10.1$ and $Q_2 = 19.9 - 7.5 = 12.4$. This gives $\lim_{t\to\infty} Q = 13.1$ mg. by equation IV.

TABLE II

ENERGIES OF ACTIVATION[a] FOR ENZYMES FROM VARIOUS SOURCES

Enzyme	$A - B$, kcal./mole	A, kcal./mole	B, kcal./mole
Pippin apple	−3.6	3.9	7.5
Mushroom juice	−3.7	11.6	15.3
Commercial mushroom	−3.4	12.1	15.5
Columbia mushroom	−3.7[b]		

[a] From least squares treatment of data in Table I with the exception of the last value. [b] From some manometric determinations of Ludwig and Nelson[8] at two temperatures.

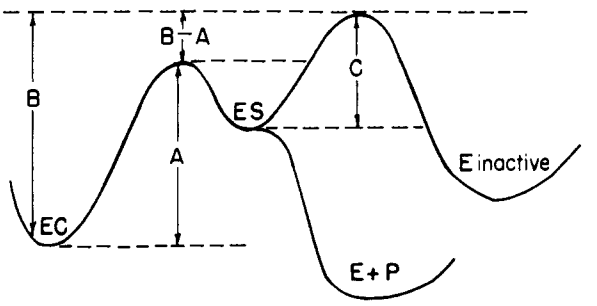

Fig. 1.

this mechanism and would very probably have a very low energy of activation such as we found, since the free radical reactions commonly have low energies of activation.

This mechanism will explain why the amount of reaction-inactivation depends on the structure of the substrate.[8] It is not open to the usual criticism of previously considered mechanisms involving free semiquinones that increasing the ascorbic acid to reduce the steady state concentration of semiquinone does not reduce the reaction-inactivation. In Fig. 1 is plotted the probable potential energy relationship for the mechanism shown above. It is evident from Fig. 1 that the energy of activation C of the actual reaction inactivation based on ES may be much smaller than the measured B and may reach the lower limit of $B - A$.

Experimental

Apple Enzyme.[9]—Apple enzyme was prepared by the method of Ponting and Joslyn,[10] modified by the addition of 1 g. of ascorbic acid per 600 g. of apples to prevent browning. The resultant solution was concentrated in a flash evaporator to approximately 10% of its original volume.[11]

Mushroom Juice.—Mushroom juice was prepared by mincing commercial mushrooms (*Psalliota campestris*) in an equal volume of ice-water and squeezing through several layers of cheesecloth. The juice was stored under toluene.

Commercial Mushroom Enzyme.—This commercial product[12] had approximately 2,000 catecholase units per ml. and 0.2% of copper on a dry-weight basis. It was used without further treatment.

Measurements of Initial Rates.—The chronometric method[2] was used without modification. At both 0 and 25° the time for oxidation of a given amount of ascorbic acid was independent of the concentration of catechol at the catechol concentration used. Determinations were made at approximately every 5° between 0 and 30°. The reaction flask and the reagents flasks were kept immersed in constant-temperature water-bath. Measurements of initial rates of a single series were corrected for slight decreases in enzyme activity over a period of a few days by daily rechecking of the initial rate at 25°. Similar corrections were made for $\lim_{t\to\infty} Q$.

Measurements of $\lim_{t\to\infty} Q$.—The ascorbic acid measurements for these determinations were made as was done in the aliquot method described previously[4] except that 1-ml. samples were taken and the ascorbic acid was determined colorimetrically.[13]

Appendix

Derivation of Equation IV[14]

Let $Q_1 = a(1 - e^{-k_1 t})$

where $\lim_{t\to\infty} Q = a = \frac{k_0 E_0}{k_1}$

$$1 - \frac{Q_1}{a} = e^{-k_1 t}$$

(8) I. Z. Eiger and C. R. Dawson, *Arch. Biochem.*, **21**, 194 (1949).

(9) Appreciation is expressed to Dr. J. D. Ponting of this Laboratory for the preparation of the apple enyzme.

(10) J. D. Ponting and M. A. Joslyn, *Arch. Biochem.*, **19**, 47 (1948).

(11) D. G. Guadagni and K. P. Dimick, *J. Agr. and Food Chem.*, **1**, 1169 (1953).

(12) Purchased from Worthington Biochemical Co., Freehold, N. J.

(13) K. A. Evelyn, H. T. Malloy and C. Rosen, *J. Biol. Chem.*, **126**, 645 (1938).

(14) The following derivation is that worked out by Dr. H. M. Hughes of the University of California Mathematics Department.

For two different reaction times, t_1 and t_2

$$k_1 t_1 = -\ln\left(1 - \frac{Q_1}{a}\right) \text{ and } k_1 t_2 = \ln\left(1 - \frac{Q_2}{a}\right)$$

$$t_2 \ln\left(1 - \frac{Q_1}{a}\right) = t_1 \ln\left(1 - \frac{Q_2}{a}\right)$$

$$1 - \frac{Q_2}{a} = \left(1 - \frac{Q_1}{a}\right)^{t_2/t_1}$$

Take $t_2/t_1 = 2$

$$1 - \frac{1}{a} Q_2 = \left(1 - \frac{1}{a} Q_1\right)^2$$

$$a = \frac{Q_1}{2 - Q_2/Q_1}$$

for points t_1, Q_1 and $2t_1$, Q_2.

Similarly it can be shown that

$$a = \frac{2Q_1}{3\sqrt{\frac{4Q_2}{Q_1}} - 3}$$

for points t_1, Q_1 and 3 t_1, Q_2 by setting $t_2/t_1 = 3$ in the above derivation.

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ALBANY, CALIF.

[CONTRIBUTION FROM THE VENABLE LABORATORY OF THE UNIVERSITY OF NORTH CAROLINA]

The Ultraviolet Absorption Spectra and the Dissociation Constants of the Monobromoquinolines

BY SAMUEL B. KNIGHT, R. H. WALLICK AND JACQUELINE BOWEN

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The ultraviolet absorption spectra of the 2-, 3-, 5-, 6-, 7- and 8-monobromoquinolines have been determined in 95% ethanol, 10% ethanol and 10% ethanol that is 0.01 molar in hydrochloric acid. The changes in intensity and the shifts in wave length of the maxima of the monobromo- and monofluoroquinolines have been correlated with molecular dimensions. The dissociation constants of the monobromoquinolines have also been determined from spectral data. Possible reasons, based on electron densities at the nitrogen atom as influenced by the various substituents, are given for the order of basic strengths.

In extending the studies done in this Laboratory on the ultraviolet absorption spectra of the monohaloquinolines,[1] the 2-, 3-, 5-, 6-, 7- and 8-monobromoquinolines have been prepared[2] and their absorption spectra have been determined in 95% ethanol, 10% ethanol, and 10% ethanol that is 0.01 molar in hydrochloric acid. The 4-bromo-isomer proved to be unstable so that measurements were impossible.

The dissociation constants of the monobromoquinolines were also determined from spectral data, and there follows a discussion of the possible reasons for results from these and other measurements that have been made

Discussion

In discussing the spectra of these compounds we shall designate the absorption bands as B for the first band and E_2 for the second, following the terminology of Braude.[3] The B band is forbidden in a symmetrical molecule but a weak absorption is found due to unsymmetrical vibrations in the plane of the ring. The E_2 band comes from π-electron migration so that two electrons are placed on one atom in the ring. The E_2 band corresponds to the N → V transition of Mulliken.[4] The intensity of this band was shown by Sklar[5] to be high for benzene (2600 Å.) since it is not forbidden if polar structures are included in the calculations.

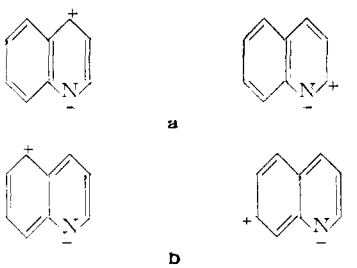

Fig. 1.

We shall consider naphthalene as the parent molecule and the quinolines as derivatives of this compound. The transition giving rise to the B band in naphthalene is N → V and is forbidden as shown from its low transition probability. This band is designated 'A–'L_b by Platt.[6] In quinolines and in the haloquinolines studied, the B band intensity is high and it follows that this is an allowed transition. Mulliken[7] states that in a compound such as pyridine where the ring symmetry is destroyed there is a transfer of the larger N → V energy to the transitions N → 'B1u and N → 'B2u which are forbidden in benzene.[5] This transfer increases the intensity of these bands.

In quinoline the B band has become an allowed N → V transition in an analogous way and ϵ_{max} shows a tenfold increase over that for naphthalene.

The ionic forms of quinoline contributing to the E_2 band are shown in Fig. 1.

These forms arise from π-electron migration from the ring onto the nitrogen atom. The nitrogen could not donate electrons to the ring since the only electrons available are the non-bonding 2s electrons. They have no nodes in the plane of the ring and therefore cannot interact with the plane of

(1) (a) W. K. Miller, S. B. Knight and A. Roe, THIS JOURNAL, **72**, 1629 (1950); (b) *ibid.*, **72**, 4763 (1950).

(2) Preparations by Arthur Roe and his students.

(3) E. A. Braude, "Chemistry of Carbon Compounds," edited by Rodd, Elsevier Publishing Co., Amsterdam, Holland, 1951, Vol. I, pt. A, Chap. 7, p. 71.

(4) R. S. Mulliken, *J. Chem. Phys.*, **7**, 20 (1939).

(5) H. L. Sklar, *ibid.*, **5**, 669 (1937).

(6) J. R. Platt, *ibid.*, **17**, 484 (1949).

(7) R. S. Mulliken, *ibid.*, **7**, 353 (1939).